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Enantiomer differentiation of α- and β-pinene in *Limonia acidissima* essential oil and its antifungal activity

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ABSTRACT

Studies on differentiation of chiral monoterpene hydrocarbons in Limonia acidissima essential oil and its antifungal activity were carried out. Enantiomeric study has revealed (-)-β-pinene as the predominant enantiomer (ee 98.6%) followed by (-)-α-pinene (ee 92.1%). The oil contained mainly phenylpropanoids marked by the presence of methyl chavicol (93.5%). Headspace-gas chromatography of isolated fresh leaves showed methyl chavicol as the major constituent in equivalent proportions as recorded in the oil. Antifungal activity of the Limonia oil was also investigated against two plant pathogenic fungi. The oil showed the best antifungal activity at 1000 ppm (based on inhibitory effect) against Curvularia lunata in comparison to Rhizoctonia solani AG4 HG III. The available reports showed the existence of racemic forms of chiral monoterpenoids; in particular, α- and β-pinene. Nonetheless, to the best of our knowledge, (-)-β-pinene is being reported for the first time as the predominant enantiomer in the essential oils. Moreover, Limonia oil can also be used in disease management of C. lunata.

INTRODUCTION

Plants constitute a major source of food for humans. Besides, many plant species are being utilized as sources of non-food industrial products such as essential oils. Plants used in pharmaceutical and perfumery preparations are also known as medicinal and aromatic plants or MAPs. Medicinal and aromatic plants are sometimes referred as medicinal, aromatic and cosmetic plants due to their applications in the field of cosmetics also [17]. Therefore, many such plant species are cultivated in smaller areas for their bio-active secondary metabolite production [7].

The intensification of the research and development programme on MAPs is attaining importance in India. However, in India, the MAPs cultivation has been severely affected by pathogenic fungi such as *Rhizoctonia solani* AG4 HGIII in *Opium*

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poppy [16], *Bipolaris australiensis* in *Senna angustifolia* [13], *Ceratobasidium* sp. in *Tagetus erecta* [14], *Pseudocercospora fuligena* in *Withania somnifera* [15] and *Cochliobolus lunatus* in *Ocimum basilicum* [18]. A significant decline in biomass production of MAPs due to these phytopathogenic fungi has been recorded. Synthetic pesticides play a major role in crop protection strategies. However, their uncontrolled application has resulted in pest resistance and hazardous environmental consequences [6].

Limonia acidissima L. Syn. *Feronia limonia* (Family: Rutaceae), also known as wood apple or katabel is an important medicinal tree, valued for its pulpy fruit and leaf-derived essential oil [5, 20]. The unripe fruit contains fruit acids, vitamins and minerals and is used mainly as a liver tonic to stimulate the digestive system and treating diarrhoea and dysentery. The fruit is also an astringent and a cardiac tonic and is also employed in the effective treatment for hiccough, sore throat and diseases of the gums. Both, the fruit pulp and the powdered rind are poultice onto bites and stings of venomous insects. While essential oil from leaves shows strong anti-bacterial activity because of its carvacrol and cyclodecandine constituents, the fruit shell has anti-fungal activity against gram positive and gram negative bacteria because of the presence of psoralene. The pulp of the *L. acidissima* fruit is also good for skin care as a moisturizer. The crushed leaf sap is also applied on itchy skins. The seed oil of this plant has a high content of unsaturated fatty acids.

Several researchers have reported the composition of *Limonia acidissima* essential oil. Most of these have reported methyl chavicol [2-4, 19] as a marker compound. However, a report from Central India showed *trans*-anethole (10.94%), thymol (24.4%) and methyl chavicol (27.2%) as marker constituents of this oil [2]. Furthermore, four out of five accessions analyzed from Western Ghats, India were found to be rich in β-pinene (63.9-76.9%) [19]. Significant variability has also been indicated in terms of compound class such as terpenes or phenylpropenes and their proportions.

In the present investigation, we report enantiomeric differentiation of chiral pairs and anti-phytopathogenic activity of essential oil isolated from *Limonia acidissima* against pathogens such as *C. lunata* and *R. solani*. To the best of our knowledge, chiral pairs are being discriminated for the first time in *Limonia* essential oil. We also report a quick method for the detection of *Limonia* leaf volatile constituents using Headspace-Gas Chromatography (HS-GC-FID).

MATERIALS AND METHODS

Plant materials and isolation of essential oils

Limonia acidissima leaves were collected from the experimental farm of CSIR-CIMAP, Lucknow. The fresh leaves were washed with water and hydrodistilled using a Clevenger-type apparatus for 4 hours. The collected oil was dried over anhydrous Na_2SO_4 and stored at 4°C prior to analysis. The average yield of the essential oil was calculated to be 0.7 % (w/w).

Headspace-Gas Chromatography analysis (HS-GC-FID)

GC analysis was done using a DB-5 capillary column (30m × 0.25 mm i.d., film thickness is 0.25 µm) on a Varian CP-3800 gas chromatograph. The column oven was programmed from 60°C to 240°C at the rate of 3°C/minute with a final hold time of 2 minutes. H_2 gas was used as a carrier gas at a constant flow rate of 1 mL/minute in the split ratio of 1:25, makeup flow (N_2 gas) at 29 mL/minute; S/SL Injector and detector (FID) temperatures were 280°C. A 1 mL headspace syringe was utilized to draw the sample vapors from the headspace vial of 20 mL capacity. About 100 mg of the fresh leaves were weighted accurately and placed in each of the three separate headspace vials. Each vial fitted with screw cap and silicone/PTFE of 18 mm 35 SHORE A septum was transported to headspace heater with the help of COMBIPAL Autosampler (CTC Analytics). Syringe was kept at 130°C with a flush time of 15 seconds. Nitrogen was used to flush the syringe. Sample incubation was carried out in a heater at 130°C with 5 minutes incubation time, incubation rpm of 300, agitator was on for 5 minutes. For sample filling, plunger fill speed was

200 µL/second with a total of 5 fill strokes and a viscosity delay of 5 seconds. Injection was done with a pre-injection delay of 0.05 seconds and a plunger injection speed of 500 µL/second.

Gas Chromatography and Mass Spectrometry

Gas Chromatography coupled with either flame ionization detection or Mass Spectrometry has been carried out as per our previously reported method [10]. Characterization was achieved on the basis of retention time, elution order, relative retention index using a homologous series of *n*-alkanes (C_6-C_{28} hydrocarbons, Polyscience Corp. Niles IL), co-injection with standards in the GC-FID capillary column (Aldrich and Fluka), TurboMass NIST 2011 libraries version 2.3.0, Wiley registry of mass spectral data 9th edition and by comparing with the mass spectral literature data [1].

Chiral Gas Chromatography

A Restek Rt™-bDEXse (30m x 0.25 mm id, 0.25 µm) fused silica capillary column was used in a Varian CP-3800 gas chromatograph with H_2 as carrier gas at 1.8 mL/minute constant flow as per our previously reported method [12].

Phytopathogens

Two phytopathogenic fungi *R. Solani* AG 4 HGIII [16] and *C. lunata* [18]were used to study the anti-fungal activity of the essential oil.

Antifungal Assay

Antifungal activity was determined by the Poisoned Food Technique (PFT) [8-9]. The experiment was repeated twice in triplicates. Percentage of the colony inhibition was calculated by the formula given as under:

$$\% \text{ of the growth inhibition} = \frac{(\text{Growth in control - growth in treatment})}{(\text{Growth in control})} \times 100$$

RESULTS AND DISCUSSION

Physico-chemical parameters

The essential oil of *L. acidissima* showed laevorotatory nature with an optical rotation value of (-) 1.01 at 20°C at 589 nm. The refractive index

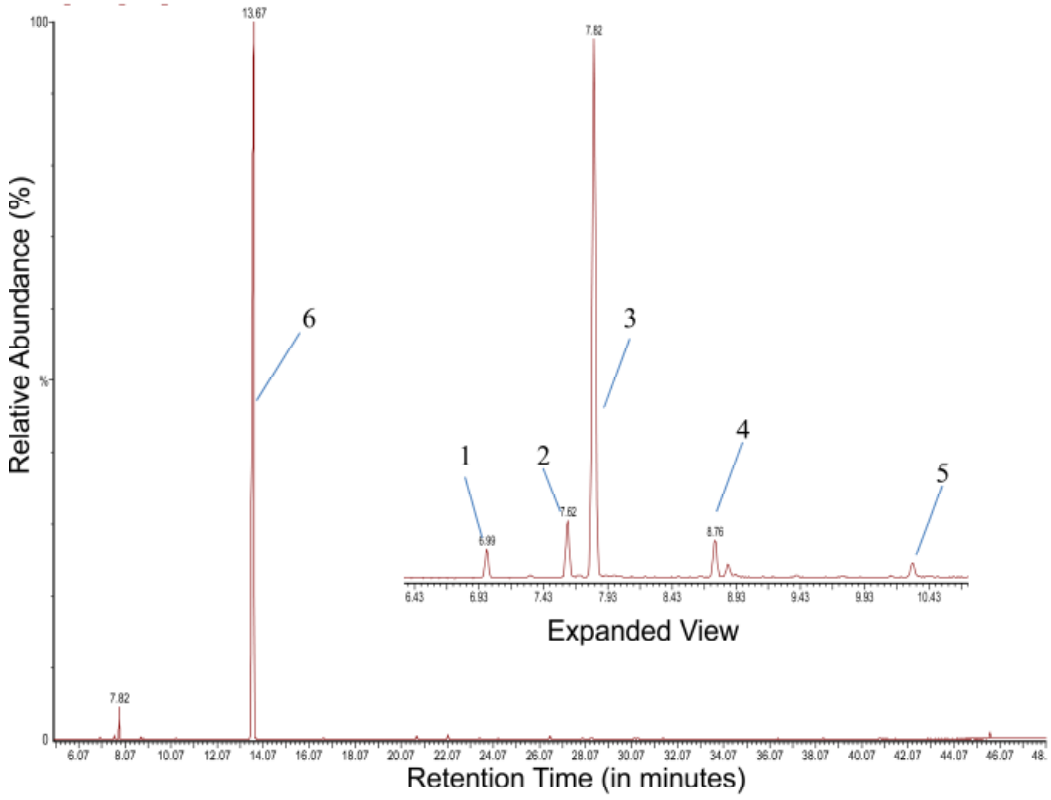

Figure 1. Total Ion Chromatogram (TIC) depicting the separation of terpenoids in *Limonia acidissima* essential oil. 1. α-pinene, 2. sabinene, 3. β-pinene, 4. limonene, 5. linalool, 6. methyl chavicol.

Table 1. Comparative proportions of constituents identified in essential oil (EO) and fresh leaves of *Limonia acidissima*

Compound	RI_a	RI_b	GC-FID % EO	HS-GC-FID % EO	Fresh leaves†
Terpenoids					
α-Pinene	932	932	0.1	0.2	0.2
Sabinene	971	969	0.2	0.4	0.2
β-Pinene	976	974	2.3	4.9	1.8
Limonene	1027	1024	0.2	0.7	0.2
Linalool	1099	1095	0.3	0.7	t
(*E*)-Caryophyllene	1418	1417	0.5	t	0.2
Phenylpropanoids					
Methyl chavicol	1205	1195	93.5	91.7	92.4
(*E*)-anethole	1283	1282	t	t	nd
Methyl eugenol	1403	1403	1.3	0.1	nd
Monoterpene hydrocarbons			2.8	6.2	2.4
Oxygenated monoterpenes			0.3	0.7	t
Sesquiterpene hydrocarbons			0.5	t	0.2
Phenylpropanoids			94.8	91.8	92.4
Total identified constituents			98.4	98.7	95.0

Notes: RI_a Retention Index on DB-5 capillary column using a homologous series of *n*-alkane (C_6-C_{28}) hydrocarbons, Polyscience Corp. Niles IL); RI_b: Adams, 2006 (Ref. 14); t: < 0.1 %; nd: not detected; † average of three samples of 100 mg fresh leaves in each HS vial.

Table 2. Enantiomeric composition (%) of chiral terpenes in essential oils of Limonia acidissima in RtTM-bDEXse phase

Compound	RI_1	Enantiomeric composition (%)	Enantiomeric excess for predominant enantiomers (%)
(+)-α-pinene	922	3.95	
(−)-α-pinene	924	96.05	92.1
(+)-β-pinene	954	0.70	
(−)-β-pinene	962	99.30	98.6
(−)-Linalool	1193	15.63	
(+)-Linalool	1203	84.37	68.7

Notes: Retention Index (RI) on RtTM-bDEXse chiral capillary column.

and relative density at 20°C were 1.5191 and 0.9641, respectively.

Essential Oil Composition

Essential oil hydro-distilled from leaves of *L. acidissima* was analyzed using enantiomeric GC-FID, GC-FID, and GC/MS techniques (Table 1). Essential oil contained more than 15 compounds, of which 9 compounds contributed to 98.4% of total oil composition. The marker constituents were methyl chavicol (93.5%), β-pinene (2.3%) and methyl eugenol (1.3%) as shown in Figure 1. The typical anise odor of this oil was attributed to the presence of a trace amount of (*E*)-anethole.

Table 3. Anti-phytopathogenic effects of Limonia acidissima essential oil

Phytopathogens	Percentage inhibition at different time (hours)				MIC value (ppm)
	24	48	72	148	
R. solani	93	55	60	-	1000
C. lunatus	100	88	77	81	1000

Headspace Gas Chromatography study of freshly plucked leaves

Headspace-GC results revealed methyl chavicol (92.4%) as the exclusive volatile constituents of leaves. The proportion was shown as an average of three accurately weight samples. Besides, β-pinene (1.8%) also appeared as single major terpene constituent in the volatile part of the plant. In contrary to the oil pattern, methyl eugenol was completely absent in Headspace analysis. Further, to confirm the volatility pattern of methyl eugenol, 100 mg of essential oil was analyzed under the same programme (Table 1). This confirms the volatile nature of methyl eugenol under developed Headspace conditions. Therefore, no detection in leaves under Headspace was possibly due to very low methyl eugenol proportion in leaves.

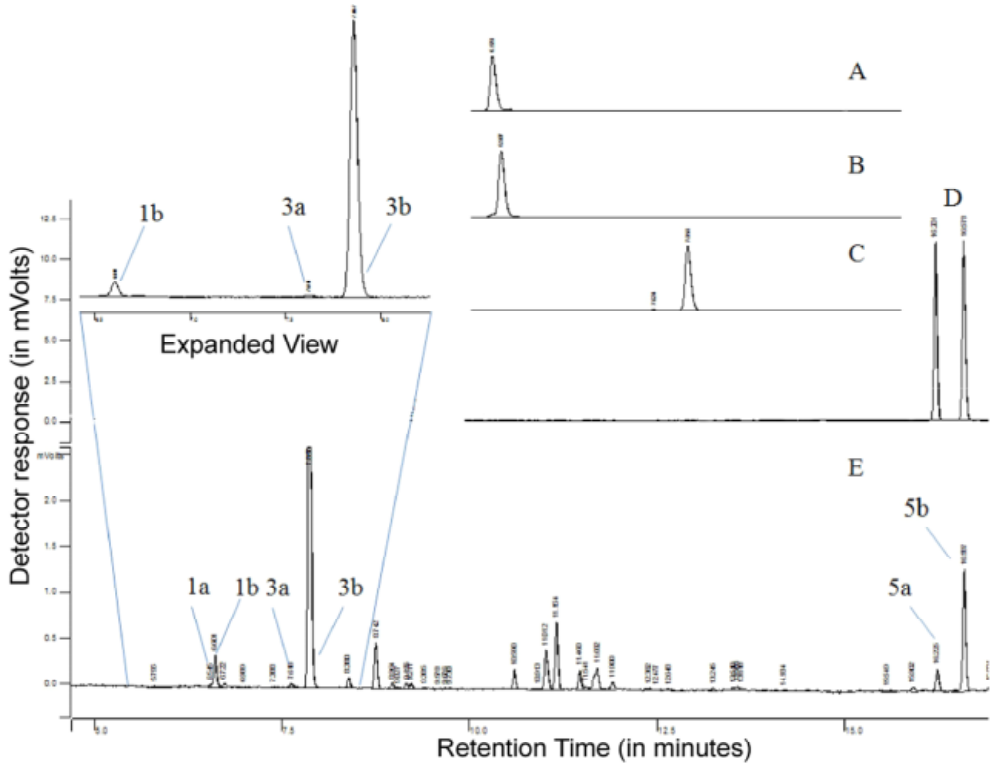

Figure 2. Gas Chromatogram showing the separation of chiral terpenoids of *Limonia acidissima* essential oil in Rt™-bDEXse chiral phase. 1a.(+)-α-pinene, 1b. (“)-α-pinene, 3a. (+)-β-pinene, 3b. (“)-β-pinene, 5a. (-)-linalool, 5b. (+)-linalool. A: standard (+)-α-pinene, B: standard (-)-α-pinene, C: standard (-)-β-pinene, D: linalool enantiomers; E: *Limonia acidissima* essential oil

Enantiomer differentiation

Separation of enantiomeric pairs has been carried out in ethylated β-cyclodextrin containing chiral column (Restek Rt™-bDEXse) using an enantioselective gas chromatography technique. Enantiomeric ratio (*er*) along with enantiomeric excess for each chiral pair was calculated (Table 2). The presence of (-)-β-pinene (*er* >99.3%) as predominant enantiomers was a characteristic feature of *Limonia* oil (Figure 2). In contrary to the published reports on chiral differentiation [11, 12], high *ee* for monoterpene hydrocarbon like (-)-β-pinene has been recorded for the first time. The elution order was further confirmed from a published report [12]. Other predominant enantiomers identified in oil were (“)-α-pinene (*er* >96) and (*S*)-(+)-linalool (*er* >84) and are listed in Table 2.

Anti-fungal activity

Anti-fungal activity results are summarized in Table 3. The data revealed nearly 81% inhibition against *C. lunatus* after one week while 55% inhibition was recorded against *R. solani* after 48 hours. The minimum inhibitory concentration (MIC) was observed to be 1000 ppm against both tested phytopathogens. Our results showed that essential oil has better anti-phytopathogenic activity against *C. lunata* in comparison to *R. solani* AG4 HGIII. Therefore, we conclude that the essential oil can be used for disease management against *C. lunata* in near future after proper *In-vivo* investigations.

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